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REVIEW

Preimplantation Genetic Diagnosis (PGD) : an overview

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Preimplantation genetic diagnosis (PDG) can be regarded as an early form of prenatal diagnosis. As the name implies, the process is performed before the implantation of the embryos. This helps the couple to start a pregnancy free of the inherited disease at risk in their family thus avoiding the problems associated with termination of pregnancy.[1] The first pregnancies with PGD were a series of couples at risk of various X-linked recessive diseases in 1989.[2] The embryos were sexed by the identification of the presence of Y-sequence in the embryonic DNA. From then on, PGD has developed and improved rapidly. At present, the cumulative data reveals referral data on 886 couples, cycle data on 1318 PGD cycles and data on 163 pregnancies and 162 babies were collected.[3] To date, reports of clinical applications of PGD include:

1. Embryonic sexing in X-linked recessive diseases. In several X-linked diseases, the responsible gene cannot be directly analysed or has not been identified. In these diseases, most of the affected individuals are male. Therefore, the identification of embryonic sex is used to select female embryos for transfer into the uterus.
2. Detecting unbalanced translocation in translocation carriers. A balanced translocation carrier may be normal but has a high risk of producing chromosomally abnormal gametes, resulting in several reproductive failures such as subfertility or recurrent abortions. PGD has been used to select normal embryos or embryos with balanced translocation for transfer into the uterus.
3. Detecting single-gene defects in the couples at risk. PGDs for several single defects have been reported such as Marfan syndrome, Huntington's chorea, myotonic dystrophy, familial adenomatous poliposis coli, cystic fibrosis, Tay-Sachs disease, Lesch-Nyhan syndrome, (-thalassemia, sickle cell disease, spinal muscular atrophy, Duchenne muscular dystrophy, Hemophilia A, Fragile X syndrome, severe combined immunodeficiency and ocular albinism I.
4. Aneuploidy screening for other kinds of women at risk in general such as in cases of advanced maternal age undergoing in vitro fertilisation (IVF) or cases of habitual abortion.
5. Aneuploidy and/or genetic screening following intracytoplasmic sperm injection (ICSI) in male infertility. There is a higher risk of abnormal Y chromosome or cystic fibrosis (in Caucasians) in men with abnormal sperm analysis.

The whole process of PGD consists of, after identification of the inherited disease at risk in the family and councelling, in vitro fertilisation (IVF), biopsy of cell(s) to be analysed and the genetic analysis.

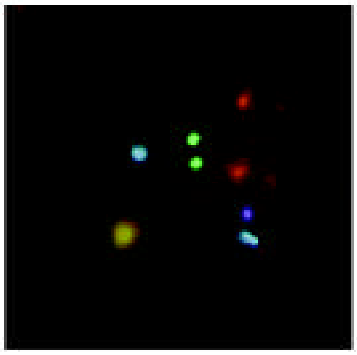

Fig. 1. FISH on a blastomere showing normal signals for chromosomes 13, 18, 21, X and Y in red, aqua, green, blue and gold respectively. The probe set was MultiVysion™ PGT from Vysis (UK) Ltd.

In Vitro Fertilisation (IVF)

To perform a PGD in a natural cycle, one cannot be certain how many embryos there are since there is always a chance of a multifetal pregnancy. In addition, a uterine lavage is needed to obtain embryos from a natural cycle. Therefore, in vitro fertilisation is incorporated into the process of PGD to ensure all embryos have been examined. IVF consists of ovarian stimulation, oocyte retrieval, and fertilisation with the sperm outside the body. Ovarian stimulation is usually accomplished by pituitary down regulation using a gonadotropin releasing hormone analogue (GnRHa) and stimulation with an external gonadotrophic hormone such as human menopausal gonadotropin (hMG), or synthetic follicle stimulating hormone (FSH). The woman must be monitored for the development of ovarian follicles using an ultrasound scan and/or blood oestradiol (E2) levels. Once there is an adequate number of suitable follicles, ovulation is stimulated by the administration of human chorionic gonadotropin (hCG) and the oocyte retrieval performed 34-36 hours after the administration just before the true ovulation takes place. Oocytes are cultured and inseminated with sperm (in case of PGD for a single gene disorder, intracytoplasmic sperm injection, ICSI, is preferred) and, after the PGD results have been achieved, unaffected embryos are transferred into the uterine cavity.

Obtaining Cell(S) for Analysis

After fertilisation, the oocyte completes metaphase II and the second polar body is extruded. The zygote then undergoes mitotic divisions. From these processes, cell(s) from several stages can be utilised for genetic analysis. Biopsy can be performed on the polar body or cells from cleavage stage or blastocyst stage embryos. The most common technique currently in use is cleavage stage biopsy.[4]

1. Cleavage stage biopsy

The biopsy is carried out on 1-2 cells from 6- to 8-cell embryos. This is usually on day 3 post-insemination. A small hole is made in the zona pellucida using acid Tyrodes solution or a laser and the blastomere(s) aspirated through the hole. After the biopsy the embryos are kept in culture and the biopsied cell(s) tested. Only unaffected embryos are replaced into the mother. The biopsy is performed at this stage because the cells are still totipotent. The remaining cells usually survive and can still undergo further development.[5] Biopsy at the 4-cell stage can retard cleavage.[6] Soussis et al.[7] reported the obstetric outcome of 16 pregnancies following PGD at the 8- to 10-cell stage (12 singleton and 4 twins), apart from 3 singleton pregnancies which were lost in first trimester, the remaining pregnancies resulted in 15 healthy babies.

Due to the high frequency of mosaicism found in this stage,[8] analysis of two blastomeres from an embryo with 8 cells or more is suggested to reduce the risk of misdiagnosis.

2. Polar body biopsy

In order to avoid embryo biopsy, some groups have used polar body. Verlinsky et al.[9] reported their experience in polar body biopsy for PGD. They tested for common aneuploidies using FISH and for some single gene disorders in 187 clinical cycles. Three-quarters of the tested cycles resulted in embryo transfers which gave rise to 38 clinical pregnancies and 12 births of an unaffected child.[10]

An important drawback of this technique is that it is prone to misdiagnosis due to crossing over between non-sister chromatids in the first meiosis, loss of a single chromatid or a chromosome from the polar body or the primary oocyte, or some errors in meiosis II. In the light of this, sequential analysis of the first and second polar body has been introduced.[11] This may reduce the chances of misdiagnosis but altogether the polar body analysis is technically and financially demanding. It may not be suitable for clinical PGD in general.

3. Blastocyst biopsy

Blastocyst biopsy is performed on day 5 or 6 when the embryonic cells become separated into the inner cell mass (ICM) and the trophectoderm (TE) with collection of fluid in the blastocoel. The technique involves a slit being made in the zona pellucida opposite the ICM and the embryo replaced into culture. As the embryo expands, the TE herniates through the slit and can be partially removed for analysis.[12] Laser biopsy has been introduced to increase the blastocyst recovery rate.[13] Ten to thirty cells from the TE can be biopsied thus giving more cells available for analysis than biopsy at other stages. This could minimise the risk of error in analysis per se or error from mosaicism which has been found to be prevalent at this stage as well[14-15] and also allows for more tests to be performed. Moreover, it should not affect the future embryo/fetus which arises from the ICM. However, with conventional embryo culture, a substantial number of human embryos arrest before the blastocyst stage, thus hampering the routine use of blastocyst biopsy clinically at present.

Genetic Analysis in Preimplantation Genetic Diagnosis

The next step in PGD is to test the biopsied cell(s). Currently, most centres performed cleavage stage biopsy from which only 1-2 cells are available. The tests carried out on one or two cells must be sensitive and accurate. At present, two important techniques used in PGD include fluorescent in situ hybridisation (FISH) for chromosome study and polymerase chain reaction (PCR) for single gene defect study.

Fluorescent in situ hybridisation (FISH)

Normally, the biopsied blastomeres are unlikely to be in metaphase so it is difficult to assess a full karyotype. The development of FISH enables a study of a certain chromosome or chromosomes on interphase nuclei. FISH has been employed for embryonic sexing in couples carrying X-linked diseases [8, 16] for the diagnosis of some chromosomal disorders in high risk couples[17] and for age-related aneuploidy.[18] However, some problems need to be considered. The major concern is mosaicism. From previous studies, embryos with normal morphology may have sex chromosome or autosome mosaicism or a completely chaotic pattern.[8,19-21] As previously mentioned, analyses of two cells from an embryo can reduce errors from this problem.

Another draw back of FISH is only a limited number of chromosomes can be studied. This is due to the limited fluorochromes available. The combination with different ratios of fluorochromes may be helpful. The analysis would, however, be difficult and inaccurate as the position of the chromosomes in interphase nuclei would hamper the visualisation of too many hybridisation. A study has applied spectral imaging on interphase lymphocytes

and was able to achieve signals from 7 chromosome pairs simultaneously.[22] However, the expected signals were obtained from 70% of interphase nuclei and various problems needed to be solved. An alternate technique is to perform 2-3 rounds of FISH on the biopsied blastomere(s).[23] Besides, structural aberrations cannot be detected by FISH without a special design of probes for a particular aberration. Several techniques have been attempted in order to obtain information of every chromosome from interphase single cells especially biopsied blastomeres. These techniques include interphase conversion and comparative genomic hybridisation (CGH).[24]

Interphase conversion results in metaphase chromosomes available for a complete karyotyping from single interphase nuclei. It involves transferring an interphase cell, such as a blastomere, into a recipient cell such as a metaphase II bovine oocyte[25] or an enucleated or intact mouse zygote.[26] The metaphase-inducing factors in the recipient cell force the transferred nucleus into metaphase that can be karyotyped either conventionally or by new technologies such as Multiplex or multifluor FISH (M-FISH) and SKY (spectral karyotyping). These techniques have been developed as advanced FISH-based methods that allow for the identification of the 24 chromosomes in a single hybridisation experiment.[27-28] They have been shown to improve the detection and defining of some subtle or complex abnormalities which are sometimes missed or difficult to diagnose by a conventional method.[29-30]

Like interphase conversion, CGH can determine a karyotype from interphase cells. The principle of CGH is the competition of hybridisation between a reference and a test DNA labelled in different colours to normal metaphases on a slide. The ratio of hybridisation between the two DNA samples on the metaphase chromosomes is determined with the aid of computer software by the different fluorochromes used to label the reference and the test DNAs. The deviation of this ratio at any location on the metaphase chromosomes suggests a gain or loss of the test DNA in that region. Also, unbalanced structural abnormalities can be detected by CGH. These abnormalities can be missed by a conventional FISH. Studies using CGH on blastomeres from cleavage stage embryos have been reported and various abnormalities involving chromosomes other than those commonly tested by FISH have been observed.[31-33] Nonetheless, CGH cannot identify ploidy abnormalities because the detection of abnormalities is based on a change in the relative binding ratio of the two genomes (reference and test DNAs) from one locus to another.[34] A loss or a gain of the whole genome (ploidy problems) will have a constant relative binding ratio and would not be detected.

Polymerase chain reaction (PCR)

PCR which enables an analysis on a small amount of DNA can be used for the diagnosis of single gene defects and triplet repeat disorders.[35] Sometimes PCR is also used for embryonic sexing but this is less informative than FISH since PCR does not give the copy number of the chromosomes.[36] Again, mosaicism can cause misdiagnosis especially for the diagnosis of dominant single gene disorders if the biopsied cell is haploid and carries the normal allele. Other concerns for PCR are contamination with exogenous DNA and allele drop out.[37,38] Because of the high sensitivity of PCR, only a minute amount of contaminating exogenous DNA can be amplified and cause misdiagnosis. Meticulous precautions should be observed to avoid contamination. There should be a specially allocated room for single cell PCR and a devoted set or sets of equipment such as pipettes, tips, PCR machine and so on for single cell PCR. The personnel should wear gloves while performing the test. The positive pressure in the PCR room will help reduce the contamination from outside. Preparation of the solutions should be performed in a laminar hood. PCR products should not be exposed in the PCR room. ICSI is also used to minimise the problem of contamination from other sperm and, because the procedure involves the removal of cumulus cells as much as possible, it also reduce the contamination

from maternal cells. On the other hand, because the sample to be analysed is from only 1-2 nuclei, failure amplification can arise easily if the PCR condition is not optimal. One of the two alleles may be less efficiently amplified than the other and this can give rise to a drop out of this allele or a preferential amplification of the other allele.

Several PCR techniques have been developed to ameliorate these problems. Nested PCR can reduce the problem of contamination and increase the efficiency of PCR. Instead of 50-60 cycles for single cells being run straightaway, the first round of PCR using "outer primer" set is performed for 20-25 cycles and the PCR product is subject to another round of 25-30 cycles of PCR using "inner primer" set. Both primer sets flank the sequence of interest, the outer primer, as the name implies, are situated lateral to the inner set in both 5' and 3' directions. A more sensitive technique, fluorescent PCR, eliminates the need to use nested PCR. The primers are labelled with a fluorescent dye and PCR product can be detected by an automated laser sequencer which is very sensitive. Less PCR cycles are needed for fluorescent PCR, reducing the chance of contamination or preferential amplification. Also, less PCR product can be detected and this has shown that allele drop out might be in fact only a less preferentially amplified allele. Finally, multiplex PCR with the primers of interest and primers for an informative polymorphic marker such as short tandem repeat (STR) can detect contamination.

Another way to minimise the chance of misdiagnosis is to repeat the test. As the available DNA is from one or two copies of the embryonic genome, attempts have been tried to amplify the whole genome so that there are more copies of the embryonic genome to be studied. Whole genome amplification can be achieved using degenerated oligonucleotide primer PCR (DOP-PCR) or primer extension preamplification PCR (PEP-PCR). Both techniques involves random amplification throughout the whole genome and, for PEP, can yield at least 30 copies of at least 78 percent of the genome.[39]

A new technology, DNA chip (DNA microarrays), enables a screening of DNA for a wide spectrum of genetic abnormalities simultaneously using the hybridisation for each abnormality from a sample. It is a small, solid supports such as microscope slides onto which thousands of cDNAs (complementary DNA) or oligonucleotides are arrayed, representing known genes or simply EST clones, or covering the entire sequence of a gene with all its possible mutations. Fluorescently labelled DNA or RNA extracted from the sample is hybridised to the array. Laser scanning of the chip permits quantitative evaluation of each individual complementary sequence present in the sample.[40] It has been suggested for neonatal screening.[41] Therefore, it is possible for PGD in the future. However, this technique raises ethical problems as the traits used for the screening may not be only medical reasons and may create the problem of "designer babies" if its use is inappropriately controlled.

Ethical Consideration

PGD is supposed to use for medical reason, preventing an affected pregnancy. However, with rapid progression in medical genetics and molecular technologies, it is now possible to examine for some nonessential characteristics including embryonic sex in cases without X-linked problem. Without a proper control, problems of "designer babies"[42] and "eugenics" can easily arise. Also, gender balance in the population may be affected or there is a potential for gender discrimination in the society. In addition, abuse of PGD can cause unnecessary medical burdens and costs for parents, and inappropriate and unfair use of limited medical resources for PGD rather than for more genuine and urgent medical needs. In several countries such as in the UK, a body or organisation comprising of relevant experts has been set up for regulating, controlling and monitoring the use of PGD to ensure the proper use and studies of this area.

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